

Amendments to the Specification:

Please replace page 1, lines 8 through 16 with the following amendment:

This is a divisional of pending application Serial No. 08/931,855 filed September 16, 1997, now United States Patent No. 6,692,751 B1, which is a continuation-in-part application of Ser. No. 563,732, filed Nov. 28, 1995, now abandoned, and of Ser. No. 272,271, filed Jul. 8, 1994, which is a continuation of Ser. No. 616,369, filed Nov. 21, 1990, abandoned, which is a continuation-in-part of Ser. No. 573,643, filed Aug. 27, 1990, abandoned; the disclosures of which are incorporated herein by reference.

Please add the following new paragraphs after the paragraph ending on page 7, line 30:

Figure 9 illustrates the nucleotide base sequence of a preferred DNA segment of the present invention that encodes portions of the structural proteins of the Hutch strain of NANBV. The base sequences are shown conventionally from left to right and in the direction of 5' terminus to 3' terminus using the single letter nucleotide base code (A=adenine, T=thymine, C=cytosine and G=guanine) with the position number of the first base residue in each row indicated to the left of the row showing the nucleotide base sequence.

The reading frame of the nucleotide sequence illustrated in Figure 9 is indicated by placement of the deduced amino acid residue sequence of the protein for which it codes below the nucleotide sequence such that the triple letter code for each amino acid residue (Table of Correspondence) is located directly below the three bases (codon) coding for each residue. The residue sequence is shown conventionally from left to right and in the direction of amino terminus to carboxy terminus. The position number for the last amino acid residue in each row is indicated to the right of the row showing the amino acid residue sequence.

Figure 10 illustrates the structure of a preferred fusion protein comprised of an amino-terminal polypeptide portion corresponding to residues 1-221 of glutathione-S-transferase, an intermediate polypeptide portion corresponding to residues 222-225 and defining a cleavage site for the protease Factor Xa, a linker portion corresponding to residues 226-234, a carboxy-terminal polypeptide portion corresponding to residues 235-308 defining a NANBV capsid antigen, and a carboxy-terminal linker portion corresponding to residues 309-315. Figure 10 also illustrates the nucleotide base sequence of a DNA segment that encodes the fusion protein illustrated therein. The nomenclature and presentation of sequence information is as described in Figure 9.

Please add the following new paragraphs after the paragraph ending on page 43, line 15:

I. Diagnostic Systems and Methods

1. Diagnostic Systems

A diagnostic system in kit form includes, in an amount sufficient for at least one assay according to the methods described herein, a NANBV structural protein or fusion protein of the present invention, as a separately packaged reagent. Instructions for use of the packaged reagent are also typically included.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

In preferred embodiments, a diagnostic system of the present invention further includes a label or indicating means capable of signaling the formation of a complex containing a recombinant protein.

As used herein, the terms "label" and "indicating means" in their various grammatical forms refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of a complex. Any label or indicating means can be linked to or incorporated in an antibody

or monoclonal antibody or used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel proteins, methods and/or systems.

The label can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labeling agents are fluorochromes such as fluorescein isocyanate (FIC), fluorescein isothiocyanate (FITC), 5-dimethylamine-1-naphthalenesulfonyl chloride (DANSC), tetramethylrhodamine isothiocyanate (TRITC), lissamine, rhodamine 8200 sulphonyl chloride (RB 200 SC), a chelate-lanthanide bound (e.g., Eu, Tb, Sm) and the like. A description of immunofluorescence analysis techniques is found in DeLuca, "Immunofluorescence Analysis", in Antibody As a Tool, Marchalonis, et al., eds., John Wiley & Sons, Ltd., pp. 189-231 (1982), which is incorporated herein by reference.

In preferred embodiments, the label is an enzyme, such as horseradish peroxidase (HRP), glucose oxidase, alkaline phosphatase or the like. In such cases where the principal label is an enzyme such as HRP or glucose oxidase, additional reagents are required to visualize the fact that an antibody-antigen complex (immunoreactant) has formed. Such additional reagents for HRP include hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine. An additional

reagent useful with HRP is 2, 2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling agents and are used illustratively herein. An exemplary radiolabeling agent is a radioactive element that produces gamma ray emissions. Elements which themselves emit gamma rays, such as  $^{124}\text{I}$ ,  $^{125}\text{I}$ ,  $^{128}\text{I}$ ,  $^{131}\text{I}$  and  $^{51}\text{Cr}$  represent one class of gamma ray emission-producing radioactive element indicating groups. Particularly preferred is  $^{125}\text{I}$ . Another group of useful labeling means are those elements such as  $^{11}\text{C}$ ,  $^{18}\text{F}$ ,  $^{15}\text{O}$  and  $^{13}\text{N}$  which themselves emit positrons. The positrons so emitted produce gamma rays upon encounters with electrons present in the animal's body. Also useful is a beta emitter, such as  $^{111}\text{indium}$ ,  $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^{32}\text{P}$ .

Additional labels have been described in the art and are suitable for use in the diagnostic systems of this invention. For example, the specific affinity found between pairs of molecules can be used, one as a label affixed to the specific binding agent and the other as a means to detect the presence of the label. Exemplary pairs are biotin:avidin, where biotin is the label; and peroxidase: anti-peroxidase (PAP), where peroxidase is the label.

The linking of labels, i.e., labeling of, polypeptides and proteins is well known in the art. For instance, antibody molecules produced by a hybridoma can be labeled by metabolic incorporation of radioisotope-containing amino acids provided as a component in the culture medium. See, for example, Galfre et al., Meth. Enzymol., 73:3-46 (1981). The techniques of protein conjugation or coupling through activated functional groups are particularly applicable. See, for example, Aurameas et al., Scand. J. Immunol., Vol. 8 Suppl. 7:7-23 (1978), Rodwell et al., Biotech., 3:889-894 (1984), and U.S. Pat. No. 4,493,795.

The diagnostic system can also include, preferably as a separate package, a specific binding agent. A "specific binding agent" is a molecular entity capable of selectively binding a reagent species, which in turn is capable of reacting with a product of the present invention but is not itself a protein expression product of the present invention. Exemplary specific binding agents are antibody molecules such as anti-human IgG or anti-human IgM, complement proteins or fragments thereof, protein A, and the like. Preferably the specific binding agent can bind the anti-NANBV antibody to be detected when the antibody is present as part of an immunocomplex.

In preferred embodiments the specific binding agent is labeled. However, when the diagnostic systems includes a specific binding agent that is not labeled, the agent is typically used as an amplifying means or reagent. In these embodiments, the labeled specific binding agent is capable of specifically binding the amplifying means

when the amplifying means is bound to a reagent species-containing complex.

The diagnostic kits of the present invention can be used in an "ELISA" format to detect the presence or quantity of antibodies in a body fluid sample such as serum, plasma or saliva. "ELISA" refers to an enzyme-linked immunosorbent assay that employs an antibody or antigen bound to a solid phase and an enzyme-antigen or enzyme-antibody conjugate to detect and quantify the amount of an antigen or antibody present in a sample. A description of the ELISA technique is found in Chapter 22 of the 4th Edition of Basic and Clinical Immunology by D.P. Sites et al., published by Lange Medical Publications of Los Altos, CA in 1982 and in U.S. Patents No. 3,654,090; No. 3,850,752; and No. 4,016,043, which are all incorporated herein by reference.

Thus, in preferred embodiments, the NANBV structural protein or fusion protein of the present invention can be affixed to a solid matrix to form a solid support that is separately packaged in the subject diagnostic systems.

The reagent is typically affixed to the solid matrix by adsorption from an aqueous medium although other modes of affixation, well known to those skilled in the art, can be used.

Useful solid matrices are well known in the art. Such materials include the cross-linked dextran available under the trademark SEPHADEX from Pharmacia Fine Chemicals (Piscataway, NJ); agarose; beads of polystyrene about 1 micron to about 5 millimeters in diameter available from Abbott Laboratories of North Chicago, IL; polyvinyl chloride, polystyrene, cross-linked polyacrylamide, nitrocellulose- or nylon-based webs such as sheets, strips or paddles; or tubes, plates or the wells of a microtiter plate such as those made from polystyrene or polyvinylchloride.

The NANBV structural protein, fusion protein, labeled specific binding agent or amplifying reagent of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry powder, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support such as the before-described microtiter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

The packages discussed herein in relation to diagnostic systems are those customarily utilized in diagnostic systems. Such packages include glass and plastic (e.g., polyethylene, polypropylene and polycarbonate) bottles, vials, plastic and plastic-foil laminated envelopes and the like.



## 2. Diagnostic Methods

The present invention contemplates any diagnostic method that results in detecting anti-NANBV structural protein antibodies or NANBV structural antigens in a body fluid sample using a NANBV structural protein, fusion protein or anti-NANBV structural antigen antibody of this invention as an immunochemical reagent to form an immunoreaction product whose amount relates, either directly or indirectly, to the amount of material to be detected in the sample. Those skilled in the art will understand that there are numerous well known clinical diagnostic chemistry procedures in which an immunochemical reagent of this invention can be used to form an immunoreaction product whose amount relates to the amount of specified antibody or antigen present in a body sample.

Various heterogenous and homogenous protocols, either competitive or noncompetitive, can be employed in performing an assay method of this invention. Thus, while exemplary methods are described herein, the invention is not so limited.

To detect the presence of anti-NANBV structural protein antibodies in a patient, a bodily fluid sample such as blood, plasma, serum, urine or saliva from the patient is contacted by admixture under biological assay conditions with a NANBV structural protein, and preferably with a fusion protein of the present invention, to form an immunoreaction admixture. The admixture is then maintained for a period of time

sufficient to allow the formation of a NANBV structural protein-antibody molecule immunoreaction product (immunocomplex). The presence, and preferably the amount, of complex can then be detected as described herein. The presence of the complex is indicative of anti-NANBV antibodies in the sample.

In preferred embodiments the presence of the immunoreaction product formed between NANBV structural protein and a patient's antibodies is detected by using a specific binding reagent as discussed herein. For example, the immunoreaction product is first admixed with a labeled specific binding agent to form a labeling admixture. A labeled specific binding agent comprises a specific binding agent and a label as described herein. The labeling admixture is then maintained under conditions compatible with specific binding and for a time period sufficient for any immunoreaction product present to bind with the labeled specific binding agent and form a labeled product. The presence, and preferably amount, of labeled product formed is then detected to indicate the presence or amount of immunoreaction product.

In preferred embodiments the diagnostic methods of the present invention are practiced in a manner whereby the immunocomplex is formed and detected in a solid phase, as disclosed for the diagnostic systems herein.

Thus, in a preferred diagnostic method, the NANBV structural protein is affixed to a solid matrix to form the solid phase. It is further preferred that the specific binding agent is protein A, or an anti-human Ig, such as IgC or IgM, that can complex with the ant-NANBV structural protein antibodies immunocomplexed in the solid phase with the NANBV structural protein. Most preferred is the use of labeled specific binding agents where the label is a radioactive isotope, an enzyme, biotin or a fluorescence marker such as lanthanide as described for the diagnostic systems, or detailed by references shown below.

In this solid phase embodiment, it is particularly preferred to use a recombinant protein that contains the antigen defined by the amino acid residue sequence shown in Figure 9 from residue 1 to residue 74, as embodied in the fusion protein as described in Example 15.

In another preferred diagnostic method, the NANBV structural protein of the invention is affixed to solid matrix as described above, and dilutions of the biological sample are subjected to the immunocomplexing step by contacting dilutions of sample with the solid surface and removing non-bound materials. Due to the multivalence of antibodies present in biological samples from infected individuals (bivalent for IgC, pentavalent for IgM) subsequent addition of labeled NANBV structural protein of the invention to this admixture will become attached to the solid phase by the sample antibody serving as a bridge between the solid phase NANBV structural protein of the

invention and the soluble, labeled NANBV structural protein. The presence of label in the solidphase indicates the presence and preferably the amount of specific antibody in the sample. One skilled in the art can determine a range of dilutions and determine therefrom a concentration of labeled antigen in the solid phase. The biological sample and the labeled NANBV structural protein of the invention can be admixed prior to, or simultaneously with contacting the biological sample with the solid phase allowing the trimolecular complex to form at the solid phase by utilizing the bridging property of bivalent or multivalent specific antibody. As a particularly useful label, biotinylated NANBV structural protein of the invention can be the labeled antigen, allowing the subsequent detection by addition of an enzyme-streptavidin, or an enzyme-avidin complex, followed by the appropriate substrate. Enzymes such as horse-radish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase or urease are frequently used and these, and other, along with several appropriate substrates are commercially available. Preferred labels with a marker which allows direct detection of the formed complex include the use of a radioactive isotope, such as, e.g., iodine, or a lanthanide chelate such as Europium.

In another embodiment designed to detect the presence of a NANBV structural antigen in a body fluid sample from a patient, the sample, (e.g. blood, plasma, serum, urine or saliva) is contacted by admixture under biological assay conditions with an anti-NANBV structural protein antibody of this invention, to form an immunoreaction admixture. The admixture is then maintained for a period of time sufficient to allow the

formation of a antigen-antibody immunoreaction product containing NANBV structural antigens complexed with an antibody of this invention. The presence and preferably amount, of complex can then be determined, thereby indicating the presence of antigen in the body fluid sample.

In a preferred embodiment, the antibody is present in a solid phase. Still further preferred, the amount of immunocomplex formed is measured by a competition immunoassay format where the antigen in a patient's body fluid sample competes with a labeled recombinant antigen of this invention for binding to the solid phase antibody. The method comprises admixing a body fluid sample with (1) solid support having affixed thereto an antibody according to this invention and (2) a labeled NANBV structural protein of this invention to form a competition immunoreaction admixture that has both a liquid phase and a solid phase. The admixture is then maintained for a time period sufficient to form a labeled NANBV structural protein-containing immunoreaction product in the solid phase. Thereafter, the amount of label present in the solid phase is determined, thereby indicating the amount of NANBV structural antigen in the body fluid sample.

Enzyme immunoassay techniques, whether direct or competition assays using homogenous or heterogenous assay formats, have been extensively described in the art. Exemplary techniques can be found in Maggio, Enzyme Immunoassay, CRC Press, Cleveland, OH (1981); and Tijssen, "Practice and Theory of Enzyme

Immunoassays", Elsevier, Amerstdam (1988).

Biological assay conditions are those that maintain the biological activity of the NANBV structural protein and the anti-NANBV structural protein antibodies in the immunoreaction admixture. Those conditions include a temperature range of about 4 C to about 45 C, preferably about 37 C, a pH value range of about 5 to about 9, preferably about 7, and an ionic strength varying from that of distilled water to that of about one molar sodium chloride, preferably about that of physiological saline. Methods for optimizing such conditions are well known in the art.

Also contemplated are the immunological assays capable of detecting the presence of immunoreaction product without the use of a label. Such methods employ a "detection means", which means are themselves well-known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel polypeptides, methods and systems. Exemplary detection means include methods known as biosensors and include biosensing methods based on detecting changes in the reflectivity of a surface (surface plasmon resonance), changes in the absorption of an evanescent wave by optical fibers or changes in the propagation of surface acoustical waves.

Another embodiment contemplates detection of the immunoreaction product employing time resolved fluorometry (TR-FIA), where the label used is able to produce a signal detectable by TR-FIA. Typical labels suitable for TR-FIA are metal-complexing agents such as a lanthanide chelate formed by a lanthanide and an aromatic beta-diketone, the lanthanide being bound to the antigen or antibody via an EDTA-analog so that a fluorescent lanthanide complex is formed.

The principle of time-resolved fluorescence is described by Soini et al., Clin. Chem., 25:353-361 (1979), and has been extensively applied to immunoassay. See for example, Halonen et al., Current Topics in Microbiology and Immunology, 104:133-146 (1985); Suonpaa et al., Clinica Chimica Acta, 145:341-348 (1985); Lovgren et al., Talanta, 31:909-916 (1984); U.S. Patent Nos. 4,374,120 and 4,569,790; and published International Patent Application Nos. EPO 139 675 and W087/02708. A preferred lanthanide for use in TR-FIA is Europium.

Reagents and systems for practicing the TR-FIA technology are available through commercial suppliers (Pharmacia Diagnostics, Upsala, Sweden).

Particularly preferred are the solid phase immunoassays described herein in Example 15, performed as a typical "Western Blot".

The present diagnostic methods may be practiced in combination with other separate methods for detecting the appearance of anti-NANBV antibodies in species infected with NANBV. For example, a composition of this invention may be used together with commercially available C-100-3 antigen (Ortho Diagnostics, Inc., Raritan, N.J.) in assays to determine the presence of either or both antibody species immunoreactive with the two antigens.

### EXAMPLES

The following examples are given for illustrative purposes only and do not in any way limit the scope of the invention.

#### 9. Production of Recombinant DNA Molecules

##### A. Isolation of NANBV Clones and Sequence Analysis

##### (1) Isolation of NANBV RNA and Preparation of cDNA

As a source for NANB virions, blood was collected from a chimpanzee infected with the Hutchinson (Hutch) strain exhibiting acute phase NANBH. Plasma was clarified by centrifugation and filtration. NANB virions were then isolated from the clarified plasma by immunoaffinity chromatography on a column of NANBV



IgC (Hutch strain) coupled to protein G sepharose. NANBV RNA was eluted from the sepharose beads by soaking in guanidinium thiocyanate and the eluted RNA was then concentrated through a cesium chloride (CsCl) cushion. Maniatis et al., Molecular Cloning: A Laboratory Manual, Maniatis et al., eds. Cold Spring Harbor, New York (1989).

The purified NANBV RNA was used as a template in a primer extension reaction admixture containing random and oligo dT primers, dNTP's, and reverse transcriptase to form first strand cDNAs. The resultant first strand cDNAs were used as templates for synthesis of second strand cDNAs in a reaction admixture containing DNA polymerase I and RNase H to form double stranded (ds) cDNAs (Maniatis et al., Supra). The synthesized ds cDNAs were amplified using an assymetric synthetic primer-adaptor system wherein sense and anti-sense primers were annealed to each other and ligated to the ends of double stranded NANBV cDNAs with T4 ligase under blunt-end conditions to form cDNA-adaptor molecules. Polymerase chain reaction (PCR) amplification was performed by admixing the cDNA-adaptor molecules with the same positive sense adaptor primers, dNTP's and TAQ polymerase to prepare amplified NANBV cDNAs. The resultant amplified NANBV cDNA sequences were then used as templates for subsequent amplification in a PCR reaction with specific NANBV oligonucleotide primers.

(2) Synthesis of Oligonucleotides For Use in NANBV Cloning

Oligonucleotides were selected to correspond to the 5' sequence of Hepatitis C which putatively encodes the NANBV structural capsid and envelope proteins (HCJ1 sequence: Okamoto et al., Jap. J. Exp. Med., 60:167-177, 1990). The selected oligonucleotides were synthesized on a Pharmacia Gene Assembler according to the manufacturer's instruction, purified by polyacrylamide gel electrophoresis and have nucleotide base sequences as shown in Table 1.

TABLE 1

Synthetic Oligonucleotides

Oligonucleotide	Putative NANBV	
<u>Designation<sup>a</sup></u>	<u>Region</u>	<u>Oligonucleotide Sequence</u>
690 (+)	Capsid 1-21	ATGAGCACGATTCCCAAACCT
693 (+)	Capsid 146-162	GAGGAAGACTTCCGAGC
694 (-)	Capsid 208-224	GTCCTGCCCTCGGGCCG
691 (-)	Capsid 340-360	ACCCAAATTGCGCGACCTACG
14 (+)	Envelope 356-374	TGGGTAAGGTCATCGATAC
15 (+)	Envelope 361-377	AAGGTCATCGATACCCT
18 (-)	Envelope 512-529	AGATAGAGAAAGAGCAAC
16 (-)	Envelope 960-981	GGACCAGTTCATCATCATATAT
<u>17 (-)</u>	<u>Envelope 957-976</u>	<u>CAGTTCATCATCATATCCCA</u>

<sup>a</sup> The oligonucleotides are numerically defined and their polarity is indicated as (+) and (-) for sense and anti-sense, respectively.

(3) PCR Amplification of NANBV cDNA

PCR amplification was performed by admixing the primer-adapted amplified cDNA sequences prepared in Example 9.A.(1) with the synthetic oligonucleotides 690 and 694 as primer (primer pairs 690:694). The resulting PCR reaction admixture contained the primer-adapted amplified cDNA template, oligonucleotides 690 and 694, dNTP's, salts (KCl and MgCl<sub>2</sub>) and TAQ polymerase. PCR amplification of the cDNA was conducted by maintaining the admixture at a 37 C annealing temperature for 30 cycles. Aliquots of samples from the first round of amplification were reamplified at a 55 C annealing temperature for 30 cycles under similar conditions.

(4) Preparation of Vectors Containing PCR Amplified ds DNA

Aliquots from the second round of PCR amplification were subjected to electrophoresis on a 5% acrylamide gel. After separation of the PCR reaction products, the region of the gel containing DNA fragments corresponding to the expected 690:694 amplified product of approximately 224 bp was excised and purified following standard electroelution techniques (Maniatis et al., Supra). The purified

fragments were kinased and cloned into the pUC 18 plasmid cloning vector at the Sma I polylinker site to form a plasmid containing the DNA segment 690:694 operatively linked to pUC 18.

The resulting mixture containing pUC 18 and a DNA segment corresponding to the 690:694 sequence region was then transformed into the E. coli strain JM83. Plasmids containing inserts were identified as lac<sup>-</sup> (white) colonies on Xgal medium containing ampicillin. pUC 18 plasmids which contained the 690:694 DNA segment were identified by restriction enzyme analysis and subsequent electrophoresis on agarose gels, and were designated pUC 18 690:694 rDNA molecules.

(5)     Sequencing of Hepatitis Clones that Encode the Putative Capsid Protein

Two independent colonies believed to contain a pUC 18 vector having the NANBV Hutch strain 690:694 DNA segment (pUC 18 690:694) that codes for the amino terminus of the putative capsid protein were amplified and used to prepare plasmid DNA by CsCl density gradient centrifugation by standard procedures (Maniatis et al., Supra). The plasmids were sequenced using <sup>35</sup>S dideoxy procedures with pUC 18 specific primers. The two plasmids were independently sequenced on both DNA strands to assure the accuracy of the sequence. The resulting sequence

information is presented as nucleotides 1-224 of Figure 9.

Plasmid pUC 18 690:694 contains a NANBV DNA segment that is 224 bp in length and when compared to the HCJ1 prototype sequence reveals two nucleotide substitutions and one amino acid residue difference in the amino terminal region of the putative capsid protein.

(6) Preparation of NANBV Clones from the 5' End of the Genome

To obtain the sequence of the NANBV Hutch genome encoding the remainder of the capsid region (Okamoto et al., Supra), the oligonucleotides 693 and 691 (described in Table 1) were used in PCR reactions. cDNA was prepared as described in Example 9.A.(1) to viral NANBV RNA from (Hutch) and used in PCR amplification as described in Example 9.A.(3) with the oligonucleotide pair 693:691. The resultant PCR amplified ds DNA was then cloned into pUC 18 cloning vectors and screened for inserts as described in Example 9.A.(4) to form pUC 18 693:691. Clones were then sequenced with pUC 18 specific primers as described in Example 9.A.(5).

Plasmid pUC 18 693:691 contains a NANBV DNA segment that is 157 bp in length and spans nucleotides 203-360 (Figure 9). The clone is not complete to the 693 primer used for generating the fragment. The sequence of this fragment

reveals three nucleotide differences when compared to the known sequence of HCJ1 and does not have any corresponding amino acid changes to the HCJ1 sequence.

To obtain the sequence of the NANBV Hutch genome encoding the putative envelope region (Okamoto et al., Supra), the oligonucleotide primers 14 through 18 (described in Table 1) were used in various combinations with NANBV Hutch RNA samples. As a source of NANBV RNA, a liver biopsy specimen from a chimpanzee inoculated with the Hutch strain at 4 weeks post-inoculation and exhibiting acute infection was used. The biopsied sample was first frozen and then ground. The resultant powder was then subjected to treatment with guanidine isothiocyanate for the extraction of RNA. RNA was extracted from the guanidium treated liver samples with phenol in the presence of SDS at 65 C. The liver samples were extracted a second time, and subjected to extraction with chloroform. The extracted RNA was precipitated at -20 C with isopropanol and sodium acetate.

The purified liver-derived RNA was used as a template in primer extension reactions with the oligonucleotides 18 and 16 to generate NANBV specific-cDNAs. To prepare cDNA to the Hutch strain amino-terminal protein coding sequences, anti-sense oligonucleotides, 18 and 16, were annealed to liver-derived Hutch RNA in the presence of dNTPs and reverse transcriptase at 42 C to form primer extension products. The first round of PCR amplification of the two cDNAs was performed by admixing the primer extension reaction products with separate pairs of

oligonucleotides 14:16 (16 primed DNA) and 14:18 (18 primed cDNA) for 30 cycles at 55 C annealing temperature. The PCR reactions were performed on the above admixture as in 9.A.(3). Aliquots from the 14:16 and 14:18 amplifications were used as templates for the second round of amplification in which the oligonucleotide pairs 15:17 and 15:18, respectively, were used as primers.

PCR reaction products from each of the primer pair reactions were analyzed by electrophoresis on low melt agarose gels. Following separation, the regions of the gel containing DNA fragments corresponding to the expected 15:17 and 15:18 amplified products of approximately 617 bp and 168 bp, respectively, were excised and eluted from the gel slices at 65 C. The resultant eluted fragments were purified by phenol and chloroform extractions. To clone the 15:17 and 15:18 fragments, the purified fragments were separately treated with the Klenow fragment of DNA polymerase and kinase for subsequent subcloning into the Sma I site of the pBluescript plasmid vector (Stratagene Cloning Systems, La Jolla, CA). Transformed E. coli DH5 colonies were analyzed for plasmid insert by restriction enzyme analysis as described in Example 9.A.(4).

pBluescript plasmid containing 15:17 or 15:18 DNA segments were purified using large scale CsCl plasmid preparation protocols. The DNA segments present in the amplified and purified plasmids were each sequenced as described in Example 9.A.(5).

The sequence of the 15:17 DNA segment is shown in Figure 9 from nucleotide 361 to 978. The sequence of the 15:18 DNA segment is also presented in Figure 9 from nucleotide 361 to 529. These two clones overlap by 168 bp of the 15:18 DNA segment.

The sequence results indicate that the 15:17 DNA segment differs by 30 nucleotides when compared to the HCJ1 sequence (Okamoto et al., Supra) and also differs by ten amino acid residues. The 15:18 DNA segment differs by seven nucleotides and by three amino acid residues when compared to HCJ1. In the overlap region, the two DNA segments differ at two nucleotide bases, namely, bases 510 and 511, where DNA segment 15:18 contains a T in place of a C and a G in place of an A, respectively, which results in a change of a serine in place of a glycine amino acid residue, at residue 171 of Figure 9. The reason for these differences is unknown and may be due to a PCR artifact.



B. Production of Recombinant DNA (rDNA) that Encodes a Fusion Protein

(1) Isolation of the 690:694 Fragment from the pUC 18 Clone and Introduction of the Fragment into the pGEX-3X Expression Vector

The pUC 18 vector containing the 690:694 DNA segment was subjected to restriction enzyme digestion with Eco RI and Bam HI to release the DNA segment having a sequence shown in Figure 9 from base 1 to base 224 from the pUC 18 vector. The released DNA segment was subjected to acrylamide electrophoresis and a DNA segment containing the 224 bp NANBV insert plus portions of the pUC 18 polylinker was then excised and eluted from the gel as described in Example 9.A.(4). The DNA segment was extracted with a mixture of phenol and chloroform, and precipitated.

The precipitated DNA segment was resuspended to a concentration of 25 ug/ml in water and treated with the Klenow fragment of DNA polymerase to fill in the staggered ends created by the restriction digestion. The resultant blunt-ended 690:694 segment was admixed with the bacterial expression vector, pGEX-3X. (Pharmacia Inc, Piscataway, N.J.) which was linearized with the blunt end restriction enzyme Sma I. The admixed DNAs were then ligated by maintaining the

admixture overnight at 16 C in the presence of ligase buffer and 5 units of T4 DNA ligase to form a plasmid of 690:694 DNA segment operatively linked to PGEX-3X.

(2) Selection and Verification of Correct Orientation of Ligated Insert

The ligation mixture containing the pGEX-3X and the 690:694 DNA segment was transformed into host E. coli strain W3110. Plasmids containing inserts were identified by selection of host bacteria containing vector in Luria broth (LB) media containing ampicillin. Bacterial cultures at stationary phase were subjected to alkaline lysis protocols to form a crude DNA preparation. The DNA was digested with the restriction enzyme Xho I. The single Xho I site, which cleaves within the 690:694 DNA segment between nucleotide position 173-178 (Figure 9), but not within the pGEX-3X vector, was used to screen for vector containing the 690:694 DNA segment.

Several 690:694 DNA segment-containing vectors were amplified and the resultant amplified vector DNA was purified by CsCl density gradient centrifugation. The DNA was sequenced across the inserted DNA segment ligation junctions by <sup>35</sup>S dideoxy methods with a primer which hybridized to the pGEX-3X sequence at nucleotide positions 614 to 633 shown in Figure 10. Vectors containing 690:694 DNA segment having the correct coding sequence for in-frame translation of a NANBV structural protein were thus identified and selected to form pGEX-3X-690:694.

(3) Structure of the Fusion Protein

The pGEX-3X vector is constructed to allow for inserts to be placed at the C terminus of Sj26, a 26-kDa glutathione S-transferase (GST; EC 2.5.1.18) encoded by the parasitic helminth Schistosoma japonicum. The insertion of the 690:694 NANBV fragment in-frame behind Sj26 allows for the synthesis of the Sj26-NANBV fusion polypeptide. The NANBV polypeptide can be cleaved from the GST carrier by digestion with the site-specific protease factor Xa (Smith et al., Gene, 67:31-40, 1988).

The nucleotide and predicted amino acid sequence of the pGEX-3X-690:694 fusion transcript from the GST sequence through the 690:694 insert is presented in Figure 10. The resulting rDNA molecule, pGEX-3X-690:694, is predicted to encode a NANBV fusion protein having the amino acid residue sequence shown in Figure 10 from amino acid residue 1 to residue 315. The resulting protein product generated from the expression of the plasmid is referred to as the NANBV capsid protein amino terminus (CAP-N).

C. Production of Recombinant DNAs (rDNAs) that Encode NANBV  
Capsid and Envelope Fusion Proteins

pGEX-3X-693:691: Plasmid pGEX-3X-693:691 was formed by first subjecting the plasmid pUC 18 693:691 prepared in Example 9.A.(6) to restriction enzyme digestion with Eco RI and Bam HI as performed in Example 9.B.(1). The resultant released DNA segment having a sequence shown in Figure 9 from base 205 to base 360 was purified as performed in Example 9.B.(1). The purified DNA segment was admixed with and ligated to the pGEX-3X vector which was linearized by restriction enzyme digestion with Eco RI and Bam HI in the presence of T<sub>4</sub> ligase at 16 C to form the plasmid pGEX-3X-693:691.

A pGEX-3X plasmid containing a 693:691 DNA segment was identified by selection Example 9.B.(2) with the exception that crude DNA preparations were digested with Eco RI and Bam HI to release the 693:691 insert. A pGEX-3X vector containing a 693:691 DNA segment having the correct coding sequence for in-frame translation of a NANBV structural protein was identified by sequence analysis as performed in Example 9.B.(2) and selected to form pGEX-3X-693:691.

The resulting vector encodes a fusion protein (GST:NANBV 693:691) that is comprised of an amino-terminal polypeptide portion corresponding to residues 1-221 of GST as shown in Figure 10, an intermediate polypeptide portion corresponding to

residues 222-225 and defining a cleavage site for the protease Factor Xa, a linker protein corresponding to residues 226-230 consisting of the amino acid residue sequence:

Gly Ile Pro Asn Ser

encoded by the nucleotide base sequence:

GGG ATC CCC AAT TCA, respectively;

a carboxy-terminal polypeptide portion corresponding to residues 231-282 defining a NANBV capsid antigen as shown by the amino acid residue sequence 69-120 in Figure 9, and a carboxy-terminal portion corresponding to residues 283-287 consisting of the amino acid residue sequence:

Asn Ser Ser END.

encoded by the nucleotide base sequence:

AAT TCA TCG TGA, respectively.

pGEX-3X-15:18: Plasmid pGEX-3X-15:18 was formed by first subjecting the plasmid Bluescript 15:18 prepared in Example 9.A.(6) to restriction enzyme digestion with Eco RV and Bam HI and the Bam HI cohesive termini were filled in as performed in Example 9.B.(1). The resultant released DNA segment having a sequence shown in Figure 9 from base 361 to base 528 was purified as performed in Example 9.B.(1). The purified DNA segment was admixed with and ligated to the pGEX-3X vector which was linearized by restriction enzyme digestion with Sma I as performed in 9.B.(1) to form the plasmid pGEX-3X-15:18.

A pGEX-3X plasmid containing a 15:18 DNA segment was identified by selection as performed in Example 9.B.(2) and crude DNA preparations were cut with Eco RI and Bam HI to release the 15:18 inserts. A pGEX-3X vector containing a 15:18 DNA segment having the correct coding sequence for in-frame translation of a NANBV structural protein was identified as performed in Example 9.B.(2) and selected to form pGEX-3X-15:18.

The resulting vector encodes a fusion protein (GST:NANBV 15:18) that is comprised of an amino-terminal polypeptide portion corresponding to residues 1-221 of GST, an intermediate polypeptide portion corresponding to residues 222-225 and defining a cleavage site for the protease Factor Xa, a linker protein corresponding to residues 226-234 consisting of the amino acid residue sequence:

Gly Ile Pro Ile Glu Phe Leu Gln Pro,

encoded by the nucleotide base sequence:

GGG ATC CCC ATC GAA TTC CTG CAG CCC,

respectively; a carboxy-terminal polypeptide portion corresponding to residues 235-290 defining a NANBV envelope antigen as shown by the amino acid residue sequence 121-176 in Figure 9, and a carboxy-terminal linker portion corresponding to residues 291-298 consisting of a amino acid residue sequence:

Trp Gly Ile Gly Asn Ser Ser END

encoded by the nucleotide base sequence:

TGG GGG ATC GGG AAT TCA TCG TGA,

respectively.

pGEX-3X-15:17: Plasmid pGEX-3X-15:17 was formed by first subjecting the plasmid Bluescript 15:17 prepared in Example 9.A.(6) to restriction enzyme digestion with Eco RI and Bam HI and the cohesive termini were filled in as performed

in Example 9.B.(1). The resultant released DNA segment having a sequence shown in Figure 9 from base 361 to base 978 was purified as performed in Example 9.B.(1). The purified DNA segment was admixed with and ligated to the pGEX-3X vector which was linearized by restriction enzyme digestion with Sma I as performed in Example 9.B.(1) to form the plasmid pGEX-3X-15:17.

A pGEX-3X plasmid containing a 15:17 DNA segment was identified by selection as performed in Example 9.B.(2) and DNA preparations were digested with Eco RI and Bam HI as indicated above. pGEX-3X vector containing a 15:17 DNA segment having the correct coding sequence for in-frame translation of a NANBV structural protein was identified as performed in Example 9.B.(2) and selected to form pGEX-3X-15:17.

The resulting vector encodes a fusion protein (GST:NANBV 15:17) that is comprised of an amino-terminal polypeptide portion corresponding to residues 1-221 of GST, an intermediate polypeptide portion corresponding to residues 222-225 and defining a cleavage site for the protease Factor Xa, a linker protein corresponding to residues 226-233 consisting of the amino acid residue sequence:

Gly Ile Pro Asn Leu Arg Ser Pro

encoded by the nucleotide base sequence:



GGG ATC CCC AAT TCC TGC AGC CCT,

respectively; a carboxy-terminal polypeptide portion corresponding to residues 234-439 defining a NANBV envelope antigen as shown by the amino acid residue sequence 121-326 in Figure 9, and a carboxy-terminal linker portion corresponding to residues 440-446 consisting of the amino acid residue sequence:

Gly Ile Gly Asn Ser Ser END

encoded by the nucleotide base sequence:

GGG ATC GGG AAT TCA TCG TGA, respectively.

pGEX-2T-15:17: Plasmid pGEX-2T-15:17 was formed by first subjecting the plasmid Bluescript 15:17 prepared in Example 9.A.(6) to restriction enzyme digestion with Eco RV and Bam HI and the Bam HI cohesive termini were filled in as performed in Example 9.B.(1). The resultant released DNA segment having a sequence shown in Figure 9 from base 361 to base 978 was purified as performed in Example 9.B.(1). The purified DNA segment was admixed with and ligated to the pGEX-2T vector (Pharmacia, INC.) which was linearized by restriction enzyme digestion with Sma I as performed in Example 9.B.(1) to form the plasmid pGEX-2T-15:17.

A pGEX-2T plasmid containing a 15:17 DNA segment was identified by selection as performed in Example 9.B.(2) and by digestion of crude DNA preparations with Eco RI and Bam HI. A pGEX-2T vector containing a 15:17 DNA segment having the correct coding sequence for in-frame translation of a NANBV structural protein was identified as performed in Example 9.B.(2) and selected to form pGEX-2T-15:17.

The resulting vector encodes a fusion protein (GST:NANBV 15:17) that is comprised of an amino-terminal polypeptide portion corresponding to residues 1-221 of GST, an intermediate polypeptide portion corresponding to residues 222-226 and defining a cleavage site for the protease Thrombin consisting of the amino acid residue sequence:

Val Pro Arg Gly Ser

encoded by the nucleotide base sequence:

GTT CCG CGT GGA TCC, respectively;

a linker protein corresponding to residues 227-233 consisting of an amino acid residue sequence:

Pro Ser Asn Leu Arg Ser Pro

encoded by a nucleotide base sequence:

CCA TCG AAT TCC TGC AGC CCT,

respectively; a carboxy-terminal polypeptide portion corresponding to residues 234-439 defining a NANBV envelope antigen, and a carboxy-terminal linker portion corresponding to residues 440-446 consisting of the amino acid residue sequence:

Gly Ile His Arg Asp END

encoded by the nucleotide base sequence

GGA ATT CAT CGT GAC TGA, respectively.

pGEX-3X-690:691: To obtain a DNA segment corresponding to the NANBV Hutch sequence sequence shown from Figure 9 from base 1 to base 360, the oligonucleotides 690:691 are used in PCR reactions as performed in Example 9.A.(6). The resultant PCR amplified ds DNA is then cloned into pUC18 cloning vectors as described in Example 9.A.(4) to form pUC 18 690:691. Clones are then sequenced with pUC 18 primers as described in Example 9.A.(5) to identify a plasmid containing the complete sequence. The resulting identified plasmid is selected, is designated pUC 18 690:691, and contains a NANBV DNA segment that is 360 bp in length and spans

nucleotides 1-360 (Figure 9).

Plasmid pGEX-3X-690:691 is formed by first subjecting the plasmid pUC 18 690:691 to restriction enzyme digestion with Eco RI and Bam HI as performed in Example 9.B.(1). The resultant released DNA segment having a sequence shown in Figure 9 from base 1 to base 360 with pUC 18 polylinker sequence is purified as performed in Example 9.B.(1). The purified DNA segment is admixed with and ligated to the pGEX-3X vector which is linearized by restriction enzyme digestion with Sma I as performed in Example 9.B.(1) to form the plasmid pGEX-3X-690:691.

A pGEX-3X plasmid containing a 690:691 DNA segment is identified by selection as performed in Example 9.B.(2). pGEX-3X vector containing a 690:691 DNA segment having the correct coding sequence for in-frame translation of a NANBV structural protein is identified as performed in Example 9.B.(2) and selected to form pGEX-3X-690:691.

The resulting vector encodes a fusion protein (GST:NANBV 690:691) that is comprised of an amin-terminal polypeptide portion corresponding to residues 1-221 of GST, an intermediate polypeptide portion corresponding to residues 222-225 and defining a cleavage site for the protease Factor Xa, a linker protein corresponding to residues 226-234 consisting of the amino acid residue sequence:

Gly Ile Pro Asn Ser Ser Ser Val Pro

encoded by the nucleotide base sequence:

GGG ATC CCC AAT TCG AGC TCG GTA CCC

respectively; a carboxy-terminal polypeptide portion corresponding to residues 235-355 defining a NANBV capsid antigen, and a carboxy-terminal linker portion corresponding to residues 356-363 consisting of the amino acid residue sequence:

Thr Gly Ile Gly Asn Ser Ser END

encoded by the nucleotide base sequence:

ACG GGG ATC GGG AAT TCA TCG TGA,

respectively.

10. Expression of the NANBV 690:694 Fusion Protein Using rDNA

The bacterial colonies which contain the pGEX-3X-690:694 plasmid in the correct orientation were selected to examine the properties of the fusion protein. Bacterial cultures of pGEX-3X-690:694 were grown to a stationary phase in the presence of ampicillin (50 ug/ml final concentration) at 37 C. This culture was inoculated at a 1:50 dilution into fresh LB medium at 37 C in the presence of ampicillin and maintained at 37 C. with agitation at 250 rpm until the bacteria reached an optical density of 0.5 when measured using a spectrometer with a 550 nm wavelength light source detector. Isopropylthio-beta-D-galactoside (IPTG) was then admixed to the bacterial culture at a final concentration of 1mM to initiate (induce) the synthesis of the fusion proteins under the control of the tac promoter in the pGEX-3X vector.

Beginning at zero time and at one hour intervals thereafter for three hours following admixture with IPTG (i.e., the induction phase), the bacterial culture was maintained as above to allow expression of recombinant protein. During this maintenance phase, the optical density of the bacterial culture was measured and 1 ml aliquots were removed for centrifugation. Each resultant cell pellet containing crude protein lysate was resuspended in Laemmli dye mix containing 1% beta-mercaptoethanol at a final volume of 50 microliters (ul) for each 0.5 OD 550 unit. Samples were boiled for 15 minutes and 10 ul of each sample was electrophoresed on a 10% SDS-PAGE Laemmli gel.

11. Detection of Expressed Fusion Proteins

To visualize the IPTG-induced fusion proteins, the Laemmli gels were stained with Coomassie Blue and destained in acetic acid and methanol. Induced proteins from separate clones were examined and compared on the basis of the increase of a protein band in the predicted size range from time zero to time three hours post-IPTG treatment. Expression of fusion protein was observed in clones that exhibited an increase from zero time of the intensity of a protein band corresponding to the fusion protein.

12. Western Blot Analysis

Samples from IPTG inductions were separated by gel electrophoresis and were transferred onto nitrocellulose for subsequent immunoblotting analysis. The nitrocellulose filter was admixed with antibody blocking buffer (20 mM sodium phosphate, pH 7.5, 0.5 M sodium chloride, 1% bovine serum albumin, and 0.05% Tween 40) for 3 to 12 hours at room temperature. Sera from humans or chimpanzees with NANB hepatitis believed to contain antibody immunoreactive with NANBV structural protein was diluted 1:500 in the antibody blocking buffer and admixed with the nitrocellulose and maintained for 12 hours at room temperature to allow the formation of an immunoreaction product on the solid phase. The nitrocellulose was then washed three times in excess volumes of antibody blocking buffer. The washes were followed

by admixture of the nitrocellulose with 50 ul of  $^{125}\text{I}$  protein A (New England Nuclear, Boston, MA) at a 1:500 dilution in antibody blocking buffer for one hour at room temperature to allow the labeled protein A to bind to any immunoreaction product present in the solid phase on the nitrocellulose. The nitrocellulose was then washed as described herein, dried and exposed to X-ray film for one to three hours at -70 C in order to visualize the label and therefore any immunoreaction product on nitrocellulose. Results of the Western blot immunoassay are shown in Tables 2 through 6. Samples prepared using pGEX-3X vector that produces control GST were also prepared as above and tested using the Western blot procedure as a control. No expressed protein (GST) was detectable having immunoreactivity with the sera shown to immunoreact with a fusion protein of this invention (GST:NANBV 690:694 fusion protein).

13. Purification of the Expressed GST:NANBV 690:694 Fusion Protein

Cultures of E. coli strain W3110 transformed with recombinant pGEX-3X 690:694 plasmids prepared in Example 10 were cultured for 3 hours following IPTG induction treatment. The cells were then centrifuged to form a bacterial cell pellet, the cells were resuspended in 1/200 culture volume in lysis buffer (MTPBS: 150 mM NaCl, 16 mM  $\text{Na}_2\text{HPO}_4$ , 4 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.3), and the cell suspension was lysed with a French pressure cell. Triton X-100 was admixed to the cell lysate to produce a final concentration of 1%. The admixture was centrifuged at 50,000 X g for 30 minutes at 4 C. The resultant supernatant was collected and admixed with 2 ml of 50% (w/v)



glutathione agarose beads (Sigma, St. Louis, MO) preswollen in MTPBS. After maintaining the admixture for 5 minutes at 25 degrees C to allow specific affinity binding between GST and glutathione in the solid phase, the beads were collected by centrifugation at 1000 X g and washed in MTPBS three times.

The GST:NANBV 690:694 fusion protein was eluted from the washed glutathione beads by admixture and incubation of the glutathione beads with 2 ml of 50 mM Tris HCl, pH 8.0, containing 5 mM reduced glutathione for 2 minutes at 25 degrees C to form purified GST:NANBV 690:694 fusion protein.

The above affinity purification procedure produced greater than 95% pure fusion protein as determined by SDS PAGE. That is, the purified protein was essentially free of procaryotic antigen and non -structural NANBV antigens as defined herein.

Alternatively, GST:NANBV 690:694 fusion protein was purified by anion exchange chromatography. Cultures were prepared as described above and cell pellets were resuspended in 8M guanidine and maintained overnight at 4 C to solubilize the fusion protein. The cell suspension was then applied to an S-300 sepharose chromatography column and peak fractions containing the GST:NANBV 690:694 fusion protein were collected, pooled, dialyzed in 4 M urea and subjected to anion exchange chromatography to form purified fusion protein.

14. Protease Cleavage of Purified GST:NANBV 690:694 Fusion Protein

Purified GST:NANBV 690:694 fusion protein prepared in Example 13 is subjected to treatment with activated Factor (Xa) (Sigma) to cleave the GST carrier from the NANBV 690:694 fusion protein (Smith et al., Supra). Seven ug of Factor X are activated prior to admixture with purified fusion proteins by admixture and maintenance with 75 nanograms (ng) activation enzyme, 8 mM Tris Hcl (pH 8.0), 70 mM NaCl and 8 mM CaCl<sub>2</sub> at 37 C for 5 minutes. Fifty ug of purified fusion protein are then admixed with 500 ng activated human factor Xa in the elution buffer described in Example 13 containing 50 mM Tris Hcl, 5 mM reduced glutathione, 100 mM NaCl, and 1 mM CaCl<sub>2</sub>, and maintained at 25 C for 30 minutes. The resulting cleavage reaction products are then absorbed on glutathione-agarose beads prepared in Example 13 to affinity bind and separate free GST from any cleaved NANBV structural antigen-containing protein. Thereafter the liquid phase is collected to form a solution containing purified NANBV structural protein having an amino acid residue sequence shown in Figure 10 from residue 226 to residue 315.

15. Immunological Detection of Anti-NANBV Structural Protein Antibodies

NANBV Hutch strain virus was injected in chimpanzees and blood samples were collected at various intervals to analyze the immunological response to NANBV by five different diagnostic assays. Chimpanzees were categorized as either being in the

acute or chronic phase of infection. The assays utilized in the evaluation of the immune response include: 1) Alanine aminotransferase (ALT) enzyme detection (Alter et al., JAMA, 246:630-634, 1981; Aach et al., N. Engl. J. Med., 304:989-994, 1981); 2) Histological evaluation for NANBV virions by electron microscopy (EM); 3) Detection of anti-HCV antibodies using the commercially available kit containing C-100-3 antigen (Ortho Diagnostics, Inc.); 4) Detection of anti-CAP-N antibodies by immunoblot analysis as described in Example 12; and 5) Detection of virus by PCR amplification as described in Example 9.

In Table 2, results are presented from ALT, EM, anti-HCV, anti-CAP-N, and PCR assays on sera from a chimpanzee with acute NANB Hepatitis.

TABLE 2

CHIMP 59 -ACUTE NANB HEPATITIS

WEEK

POST				ANTI	PCR
<u>INNOC</u>	<u>ALT</u>	<u>EM</u>	<u>ANTI HCV</u>	<u>CAP-N<sup>1</sup></u>	<u>690-691</u>
8	26	++	-	-	-
10	26	+	-	+	-
12	107	+	-	+	-
14	115	+	+	+	-
16	26	+	+	+	+
18	17	ND	+	+	(+)
20	11	ND	+	+	-

The results in Table 2 show immunoreaction between fusion protein and anti-NANBV structural protein antibodies in the sera tested. Furthermore, seroconversion is detectable by the immunoassay using fusion protein containing capsid antigen at times earlier than when the same sera is assayed in the C-100-3-based immunoassay.

---

<sup>1</sup> A plus (+) indicates immunoreaction was observed between admixed serum and the fusion protein, designated "CAP-N" because it corresponds to the amino terminal of the putative NANBV capsid protein, using the Western blot immunoassay described in Example 12.

In Table 3, results are presented from ALT, anti-HCV and anti-CAP-N assays on sera collected from a human with definitive NANB Hepatitis.

TABLE 3

NYU - 169 - DEFINITIVE NANB HEPATITIS

<u>Week Post Infect</u>	<u>ALT</u>	<u>Anti HCV</u>	<u>Anti CAP-N</u>
2	34	-	-
6	8	-	-
10	150	-	-
12	118	-	-
14	183	-	+
16	317	-	+
19	213	-	+
23	53	-	+

The results in Table 3 show that in the human series 169 seroconversion sera samples, the CAP-N antigen present in the fusion protein detects NANBV-specific antibodies as early as 14 weeks post inoculation, whereas the C-100-3 based immunoassay does not detect any anti-NANBV antibody at the times studied.

In Table 4, results are presented from ALT, EM, anti-HCV, and anti-CAP-N assays on sera from a chimpanzee with a self limited infection presented.

TABLE 4

CHIMP 213 - SELF LIMITED INFECTION

Week Post				Anti
<u>Innoc</u>	<u>ALT</u>	<u>EM</u>	<u>Anti HCV</u>	<u>CAP-N</u>
4	24	+	-	+
6	34	+	-	+
8	38	+	-	+
13	28	ND	-	+
16	25	ND	-	+
18	23	ND	+	+
20	25	-	+	+

The results in Table 4 show that the CAP-N antigen detects anti-NANBV antibodies earlier than the C-100-3 antigen when using sera sampled during the course of a self-limiting NANBV infection.

In Table 5, results are presented from ALT, anti-HCV and anti-CAP-N assays on sera from a chimpanzee that converted from an acute infection profile to a chronic one.

TABLE 5

CHIMP 10 - ACUTE/CHRONIC NANB HEPATITIS

<u>Symptoms</u>	<u>Week Post Innoc</u>	<u>Peak ALT</u>	<u>Anti HCV</u>	<u>Anti CAP-N</u>
acute	2	223	-	+
chronic	40	223	+	+
chronic	42	223	+	+
chronic	44	223	+	+
chronic	51	223	+	-

The results in Table 5 indicate that the CAP-N antigen preferentially detects anti-NANBV antibodies in acute stages of NANBV infection.

In Table 6, results are presented from ALT, EM, anti-HCV and anti-Cap-N assays on sera collected at various intervals from several chimpanzees with acute or chronic NANB Hepatitis.

TABLE 6

ADDITIONAL ACUTE SERA

<u>Week Post Innoc</u>	<u>Week Post Alt Elev</u>	<u>Peak ALT</u>	<u>Anti HCV</u>	<u>Anti CAP-N</u>
2	+1	73	-	+
14	+2	66	-	+
6	+2	197	-	+
11	+1	151	-	-
8	+4	125	-	+
15	+1	82	-	+
12	-4	73	ND	+

ADDITIONAL CHRONIC SERA

156	+131	110	+	+
156	-	89	+	+
160	-	89	+	+

The results in Table 6 indicate that the CAP-N antigen more often detected anti-NANBV antibodies in sera from acutely infected individuals than did the C-100-3 antigen.



The results of Tables 2-6 show that the NANBV structural protein of the invention, in the form of a fusion protein containing CAP-N antigen and produced by the vector pGEX-3X-690:694, detects antibodies in defined sera conversion at times in an infected patient or chimpanzee earlier than detectable by present state of the art methods using the C-100-3 antigen. In addition, the results show that CAP-N antigen is particularly useful to detect acute NANBV infection early in the infection.

Taken together, the results indicate that patients infected with NANBV contain circulating antibodies in their blood that are immunospecific for NANBV antigen designated herein as structural antigens, and particularly are shown to immunoreact with the putative capsid antigen defined by CAP-N. These antibodies are therefore referred to as anti-NANBV structural protein antibodies and are to be distinguished from the class of antibodies previously detected using the NANBV non-structural protein antigen C-100-3,